Chapter 32

Chlorophyll Sensitizers in Photodynamic Therapy

Alexander S. Brandis[1], Yoram Salomon[2] and Avigdor Scherz[1]*
Departments of [1]Plant Sciences and [2]Biological Regulation,
The Weizmann Institute of Science, Rehovot, Israel

*Author for correspondence, email: avigdor.scherz@weizmann.ac.il

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Summary

Photodynamic therapy (PDT) has proved to be a viable and interesting alternative to currently used less selective methods for palliative care of cancer and, in a limited number of cases, for curative treatment. Still, in spite of impressive progress and a few approvals for clinical applications, the great potential of PDT has not yet been fully realized because of current deficiencies of applied sensitizers and of applied treatment strategies. Introduction of chlorophyll- and bacteriochlorophyll-derived sensitizers is expected to markedly change this situation in the coming decade. In this and the following chapter we provide an updated summary of these new sensitizers, their syntheses, relevant characteristics and pharmaceutical activity in vitro and in vivo. The first chapter is focused on the general principles of photodynamic therapy with particular emphasis on the vascular-targeted approach to treatment. A general introduction is followed by a comprehensive description of chlorophyll based sensitizers. The following chapter (Chapter 33) is focused on the use of bacteriochlorophyll derivatives.

I. Introduction

A. Definition and Current Strategy of Photodynamic Therapy

Photodynamic therapy (PDT) of cancer is a relatively new method of treatment whereby non-toxic drugs (sensitizers) and non-hazardous visible and near infrared light (VIS/NIR) combine to generate cytotoxic reactive oxygen species (ROS) at a selected treatment site. The application of PDT in tumor care has been motivated by the quest for a treatment that is at least as effective but more selective than radio- and chemo-therapies, thus minimizing side effects. Current PDT aims at directly killing tumor cells and typically consists of five steps.

1. Administration of a photosensitizer, usually intravenously (i.v.).

2. A delay period that allows for retention or accumulation of photosensitizers in the target tissue.

Abbreviations: ALA – 5-aminolevulinic acid; AMD – age-related macular degeneration; BChl – bacteriochlorophyll; BChn – bacteriochlorin; BOLD MRI – blood oxygen level-dependent magnetic resonance imaging; BPP – bacteriopurpurin; BSA – bovine serum albumin; Chl – chlorophyll; Chl-Ser – chlorophyllide *a* L-serine ester; Chn – chlorin; CNV – choroidal neovascularization; EC_{50} – median effective concentration; HDL – high-density lipoprotein; HpD – hematoporphyrin derivatives; HPMA – N-(2-hydroxypropyl)methacrylamide; HPPH – [3-(1-hexyloxyethyl)]-pyropheophorbide; i.p. – intraperitoneally; i.v. – intravenously; IC_{50} – median inhibitory concentration; IgG – immunoglobulin G; ISC – intersystem crossing; LD_{50} – median lethal dose; LDL – low-density lipoprotein; NPe_6 – mono-L-aspartyl Chn e_6; PDT – photodynamic therapy; Pheide – pheophorbide; PP – purpurin; pyro-Pheide – pyropheophorbide; QSAR – quantitative structure-activity relationship; ROS – reactive oxygen species; TOOKAD® – [Pd]-bacteriochlorophyllide *a*; ϕ_Δ – quantum yield of singlet oxygen

3. Illumination of the target tissue (transcutanously or interstitially via optical fibers for inner organs) with consequent local generation of cytotoxic ROS.

4. Development of tumor necrosis and tumor eradication.

5. Tissue remodeling and healing.

New sensitizers have usually been designed to achieve preferential accumulation in the tumor cells while photoexcitation techniques have been developed to irradiate relatively deep tumor tissue thus expanding the usefulness and efficacy of PDT. Different porphyrinoids have been suggested as the preferred PDT sensitizers (Pandey and Zheng, 2000; Osterloh and Vicente, 2002), although cyclic and long-chain polyenes with a significant light absorption in the UV-VIS such as indocyanines and hypericin were also considered (Delaey et al., 2000; Chen et al., 2002; Kassab, 2002). For several decades, research and pre-clinical efforts have focused on hematoporphyrin derivatives (HpD) and other peripherally substituted porphyrins of (near) D_{4h} symmetry (Henderson and Dougherty, 1992; Dougherty et al., 1998; Bonnett, 1999; Pandey and Zheng, 2000; Ackroyd et al., 2001).

The preferential accumulation of hematoporphyrin derivatives in tumors relative to normal tissues, the formation of cytotoxic ROS, and necrotic or apoptotic processes that culminate in tumor eradication, were promising in early PDT development (Dougherty, 1987; Jori, 1992, 1996; Dougherty et al., 1998; Ronn, 1999); however, none of the clinically or pre-clinically used sensitizers have yet shown sufficiently high accumulation in the malignant tumor cells to enable optimally-selective and efficient treatment (Bonnett, 1999). In addition, the high attenuation of light in the UV-VIS domain, required for activation of tetrapyrrole senstitizers with D_{4h}-symmetry within animal tissues, has prevented treatment of massive tumors. Thus, the current clinical targets of PDT include relatively shallow malignant and benign tumors, choroidal neovascularization (CNV) in age-related macular degeneration (AMD), atherosclerotic lesions, as well as bacterial and viral infections. In addition, the photodynamic effect can be used as a subsidiary but method selectively for light-enhanced delivery of a drug to the body area/organ to be treated (Selbo et al., 2002).

Over the past decade, the use of PDT has increased significantly, mainly in cancer and AMD treatment. Protoporphyrin-based photosensitizers (tradenames: Photofrin®, Photosan®, Photoheme®, HpD®, Levulan®, Visudyne®) have been approved for clinical use, and successfully employed in PDT in many countries.

In spite of the impressive progress, the full potential of PDT has not yet been realized for the following reasons:

(1) the spectroscopic properties of the clinically used sensitizers only allow for sensitization of shallow tumors,

(2) the pharmacological properties of the current sensitizers (i.e., lack of sufficient specificity for the tumor cells) together with the treatment strategy result in eradication of both tumor and non-tumor tissues within the illuminated zone, and finally

(3) the retention of sensitizer in non-tumor tissues (e.g. skin) leads to prolonged cutaneous toxicity.

The disadvantages of currently-used drugs have long been known and have led to an extensive search for new sensitizers with superior spectroscopic properties.

As they were selected by evolutionary processes to perform VIS-NIR light-harvesting with consequent radical generation in photosynthesis, Chls and BChls have emerged as attractive alternative PDT sensitizers. However, because of their superior photophysics combined with a low chemical stability, Chls and BChls are rapidly degraded when exposed to light outside of their native and protective environment in the transmembrane proteins of light-harvesting complexes and photoreaction centers of chloroplasts. To overcome this limitation, major research efforts have focused on producing Chl- and BChl-like molecules that are sufficiently stable to enable light-induced radical generation deep within animal tissues and, preferentially, with higher selectivity for tumor cells than currently used sensitizers. Recently, an extensive description and screening of different Chl/BChl-based sensitizers has been published (Pandey and Zheng, 2000).

In this and the following chapter, we shall focus respectively on Chl- and BChl-derived PDT sensitizers. Each section will highlight features related to sensitizer preparation, its photochemical and

photophysical properties, in vitro screening and in vivo targets. The state of clinical development will be described at the end of each chapter.

Finally, we shall discuss current PDT treatment strategies and suggest an alternative approach made possible by new BChl-based sensitizers. The last review, dedicated to Chl and BChl sensitizers, was published more than a decade ago (Spikes and Bommer, 1991).

B. Guidelines for Selecting New Sensitizers

In searching for new and better PDT reagents, the following features have been generally accepted as criteria for optimal photosensitizers (Jori, 1996; Bonnett, 2000). These are especially relevant for PDT reagents synthesized from natural compounds such as Chl and BChl.

1. Chemical Purity

There is a consensus that new sensitizers should be chemically pure compounds unlike the composite sensitizer Photofrin®. A pure, single compound can be localized and targeted more efficiently and it is easier to estimate its sensitizing efficiency as well as its pharmacological and photochemical properties. Thus treatment protocols can be more rationally designed with readily identifiable quantitative structure-activity relationships (QSAR) (Dougherty et al., 1998; Pandey and Zheng, 2000; Macdonald and Dougherty, 2001 and refs. therein).

2. Significant Absorption at Long Wavelengths (>650 nm)

New sensitizers should preferably have strong electronic transition intensities in the range of 650–850 nm, where light penetration into animal tissue is maximized. At shorter wavelengths, endogenous pigments and light scattering substantially attenuate the photon flux to the tissue-impregnated sensitizers. At longer wavelengths, the energy transfer from the excited sensitizer becomes insufficient to transform oxygen into the excited singlet state (Moan, 1990). Further, the increased absorption of light by water molecules (λ >900 nm) reduces the effective dose of light and enhances thermalization (Macdonald and Dougherty, 2001).

3. High Quantum Yield of Reactive Oxygen Species

The type and quantity of ROS generated by an excited sensitizer determines its potential PDT efficacy. ROS generation is initiated from the triplet state of the excited sensitizer (^1T_S), which is populated by intersystem crossing (ISC) from the lowest excited singlet state (^1S_S) during its lifetime which is a few nanoseconds in the best cases. Energy or electron transfer from ^1T_S to molecular oxygen may result in:

(1) electron transfer from the excited sensitizer to the ground state oxygen, forming a superoxide radical (Type I processes) or

(2) relaxation of the sensitizer to the ground state ^0S_S with concomitant excitation of molecular oxygen to the excited singlet state (Σ^1O_2) (Type II processes) (Foote, 1968; Henderson and Dougherty, 1992; Pandey and Zheng, 2000; Macdonald and Dougherty, 2001).

The quantum yield for a particular ROS type depends on the nature of the sensitizer, the availability of oxygen, and the reaction environment. Heavy atoms and side groups increase the yield of the intersystem crossing and the amount of resulting singlet oxygen. The relative redox potentials of the excited sensitizer and concentrations of the molecular oxygen in the particular reaction environment determine the yield of electron transfer (Type I). The fate of the ROS and their cytotoxicity strongly depend on the site of their generation. This is mostly relevant to the oxygen radicals, which can be more reactive than $^1O_2$, and therefore possess a shorter lifetime than the excited singlet oxygen. Lipids, proteins and transition metals at low redox states may convert ROS to even more reactive forms that can initiate radical chain reactions (Halliwell and Gutteridge, 1990; Henderson and Dougherty, 1992). With most available sensitizers, Type II processes are generally believed to be the major pathway involved in tissue destruction, (Henderson and Dougherty, 1992; Pandey and Zheng, 2000; Bonnett, 2002); however, Type I processes may be highly relevant to PDT with Chl/BChl derived sensitizers.

4. No Dark Toxicity and No Undesired Phototoxicity in Skin, Eyes and Mucous Epithelia

Rapid clearance of the photosensitizer is desired, with no dark toxicity or mutagenic activity of the sensitizer or its degradation products. With a hydrophobic sensitizer, the carrier systems need to be clinically safe (Jocham, 1998).

5. Stability and Ease of Packaging

Before administration, the photosensitizer should have a long-term stability and shelf life; however, limited stability in vivo and under irradiation could be desirable to reduce the damage to normal tissue at threshold concentrations (Moan, 1986; Boyle and Potter, 1987; Svaasand and Potter, 1992). Water-soluble substances are favored.

6. Selectivity of Damage and Localization

Selectivity is important for PDT treatments which require the presence of light, sensitizer and oxygen to produce local cytotoxicity in the target tissue; thus a well-defined zone of destruction is provided while preferably maintaining differentiation between normal tissue cells and those of target tumor cells.

Tumor necrosis can be generally induced by: (i) direct cell killing; (ii) hypoxia caused by vascular shutdown, and (iii) immune effects. Here we shall briefly refer to the current status in PDT research.

(i) Current research is aimed at developing new PDT reagents that preferentially accumulate in tumor cells. Increasing the reagent's hydrophobicity is generally believed to enhance its accumulation in the neoplastic cells (Kozyrev et al., 1996a; Pandey et al., 1996c; Henderson et al., 1997; Pandey and Zheng, 2000). While better accumulation and higher efficacy of more lipophylic sensitizers was demonstrated in cultured tumor cells (Pandey et al., 1997a; Zheng et al., 2001b), no therapeutic benefit of these sensitizers was ever demonstrated in vivo. Indeed, the proven role of the antivascular effect of PDT (Henderson and Dougherty, 1992; Fingar, 1996; Regillo, 2000; Zilberstein et al., 2001; Schreiber et al., 2002; Koudinova et al., 2003) raises questions about the usefulness of such an approach.

(ii) The role of vascular shutdown in induction of tumor necrosis is now highly appreciated and suggests several new treatment approaches (Folkman, 1995; Schnitzer, 1998). Numerous experiments have indicated that tumor regression and cure after most PDT treatments involve occlusion and/or perforation of blood vessels (Boyle and Dolphin, 1996; Dougherty et al., 1998; Pandey and Zheng, 2000; Macdonald and Dougherty, 2001). This effect is more profound in treatment protocols that involve short intervals between drug administration and irradiation, and/or more hydrophilic sensitizers (Krammer, 2001). Further, relative response differences between the vascular bed of the tumorous and the normal tissue may provide the key for treatment selectivity as suggested by several recent studies (Ferrario et al., 1992; Roberts and Hasan, 1992; McMahon et al., 1994; Zilberstein et al., 2001; Dolmans et al., 2002a; Gross et al., 2003; Koudinova et al., 2003). Thus, the synthesis of specific vascular-directed PDT reagents may become an attractive option in future development of new sensitizers.

(iii) Systemic tumor response to PDT involving the immune system and other mechanisms that results in metastatic tumor regression has been reported by several laboratories (Canti et al., 2002; Schreiber et al., 2002). This may provide the basis for new PDT treatments that are no longer limited to the treatment of single local tumors. However, it is not yet clear how to design new sensitizers for such broader treatment strategies.

In the following sections, we shall review the Chl derivatives, highlighting the above-mentioned guidelines. BChl derivatives are treated in Chapter 33 (Brandis et al.).

II. Photosensitizers Derived from Chlorophyll *a*

A. Chlorophyll a

1. General Description and Chemistry

Natural Chl *a* (see formula in Chapter 1, Scheer) is a dihydroporphyrin with a fifth, isocyclic ring. Chl *a* can be extracted and purified with high yield and purity from practically inexhaustible plant and algal resources. Chl *a* without the admixture of

other chlorophylls can be obtained from biomass of the blue-green alga, *Spirulina*. Chl *a* has a high extinction coefficient at 660 nm ($\varepsilon \sim 10^5\ M^{-1}\ cm^{-1}$) and good singlet oxygen production ($\phi_\Delta = 0.57$ in CCl_4) (Krasnovsky Jr et al., 1990). However, Chl *a* is water-insoluble and very unstable, undergoing oxidative degradation in the presence of light, acid, bases and alcohols, and demetalation in the presence of acids. Therefore, Chl *a* is not suitable for pharmaceutical application but may provide a suitable source for the synthesis of new sensitizers that comply with the pharmaceutical requirements (for review see Spikes and Bommer, 1991). Such sensitizers should be derived by modifications that do not alter the π-electron system which provides the optical spectra. They should allow, on the other hand, modifications to the redox potential and the overall reactivity of the compound. Metal incorporation and modification of the C-3 vinyl substituent or the C-13[1] carbonyl on the isocyclic ring E will modify both the reactivity and stability of the Chls and should be carefully designed.

2. Pre-clinical Studies and Efficacy

No extensive studies have been conducted for determining the effectiveness of Chl *a* as a PDT agent.

B. Chlorophyllide a and Derivatives

1. General Description and Chemistry

Chlide *a* is a produced from Chl *a* by hydrolysis of the C-17[3] phytyl ester, which can be accomplished enzymatically with chlorophyllase. Enzymatic (Fiedor et al., 1992, 1996) and catalytic (Scherz et al., 1994) esterification of Chlide *a* as well as enzymatic (Fiedor et al., 1996; Scherz et al., 1994) and non-enzymatic transesterification (Scheer et al., 2001) of Chl *a* with different amino acids (e.g., serine, tyrosine), peptides and proteins significantly enhanced the pigment's hydrophilicity. The excited Chlide *a* generates singlet oxygen with a ϕ_Δ of about 0.3–0.4 (Fiedor et al., 1993).

Targeting. Further modification of the Chlide *a* propionic acid residue at C-17 via chemically activated amidation enabled conjugation with peptides, hormones and proteins as cell-specific ligands; for example, conjugation with melanocyte stimulating hormones for site-specific PDT of melanoma (Scherz et al., 1994).

2. Pre-clinical Studies and Efficacy

The L-serine derivative (Chl-Ser) showed 100-fold higher photocytotoxicity in M2R melanoma cell cultures than Photosan® (Rosenbach-Belkin et al., 1996). In vivo, the water-soluble Chl-Ser was excreted from the normal tissues within 72 h, but clearance was considerably and favorably retarded from tumor tissues (Rosenbach-Belkin et al., 1996), thus providing low skin phototoxicity. Its photodynamic activity, as tested in vivo on melanotic M2R melanoma tumors, was highly significant (Tregub et al., 1992; Scherz et al., 1994).

C. Pheophorbide a and Derivatives

1. General Description and Chemistry

Pheide *a* is the free-base analogue of Chlide *a*, easily obtained from Chl *a* by acidic elimination of the phytol side chain and central Mg. Its extinction coefficient near 660 nm is about 2/3 that of Chl *a,* but it has a higher dark- and light-stability, with ϕ_Δ of about 0.6 (Krasnovsky Jr et al., 1990; Fernandez et al., 1997). To examine the effect of pigment lipophilicity on the biological activity of Pheide *a*, three families of compounds with increasing n-octanol/water partition coefficients were synthesized: (a) 3-(1-alkoxyethyl) ether derivatives of Pheide *a* methyl ester (Pandey et al., 1991a); (b) esters with a longer alkyl chain (Wongsinkongman et al., 2002); and, (c) amides, in which the carboxylic group was linked to amino alkyls of various lengths and terminal functional groups (e.g. hydroxyl, amine, carboxyl, sulfonyl, sulfhydryl, and phosphoryl) (Dagan et al., 1995). It was found, in the presence of plasma, that the methyl esters of the tested 3-(1-alkoxyethyl) ether derivatives were susceptible to hydrolysis, probably by plasma esterases, whereas the ether bonds remained stable (Pandey et al., 1991a; Bellnier et al., 1993). Further, these compounds were found to slowly convert, at room temperature, into the more stable pyro-Pheides by cleavage of the methoxycarbonyl group at C-13[2] of the isocyclic ring (Pandey et al., 1996c). De-esterification of 3-(1-hexyloxyethyl) and 3-(1-heptyloxyethyl) ethers of the pyro-Pheide *a* methyl ester was carried out with LiOH-THF*(Pandey et al., 1996c). The ϕ_Δ remains at a value of 0.5 (Pandey et al., 1996c), close to that of the parent compound. The pyro-Pheide *a*

*LiOH-THF also promoted allomerization of pyro-Pheide (Kozyrev et al., 1998b).

derivative was further modified to determine the effect of steric hindrance, unsaturation and electron withdrawing capacity of introduced groups on the biologial activity of the parent compound. The tested substitutions included (a) 1-alkoxyethyl (secondary) and alkoxymethyl (primary) ethers of different chain lengths, formyl or ethyl instead of the vinyl group at C-3; (b) thiocarbonyl or methylene instead of the carbonyl at C-13^1; (c) 1-heptyloxyethyl ether instead of ethyl at C-8; (d) di-*t*-butyl aspartyl instead of methyl ester at C-17^3; and (e) the formyl group or alkoxymethyl (primary) ethers with different chain lengths at the C-20 position (Pandey et al., 1992c, 1996a,c). The replacement of the C-3 vinyl with the formyl group shifted the absorption maximum to 690 nm. The reduction of the C-13^1 oxo group in the pyro-Pheide *a* derivatives to CH_2 resulted in a blue shift to 648 nm (Pandey et al., 1996c), whereas the formation of alkyl ethers and pyro-compounds retained optical properties similar to those of the initial Pheide *a*.

Water-soluble derivatives of pyro-Pheide *a* were synthesized by substituting the vinyl with either the 2-carboxymethyl or 2-hydroxyethyl groups together with amidation of the propionic acid residue with glycine or aspartate. Being water-soluble (>5 mg kg^{-1} in 0.9% saline solution), these compounds underwent disaggregation with the addition of human serum albumin (Ando et al., 1991b). For testing the contribution of hydroxyl residues to Pheide hydrophilicity, the oxo, methoxycarbonyl and propionic acid groups of Pheide and pyro-Pheide were reduced to the corresponding mono-, di- and tri-ols. Although the resulting compounds were amphiphilic with opposing hydrophilic and lipophilic sites in the molecule, their water solubility was quite low (Bonnett et al., 1992, 1994). Recently, new water-soluble glucose and galactose derivatives of Pheide and pyro-Pheide were synthesized for use in PDT (Aksenova et al., 2000, 2001). Amidation of pyro-Pheide and its Zn complex with 2-trimethylammonium ethyleneamine led to cationic water-soluble photosensitizers, which efficiently induced DNA cleavage when irradiated at 690 nm: singlet oxygen and electron transfer mechanisms were invoked for the metal free compound and Zn complex, respectively (Mansouri et al., 1994). A similar cationic water-soluble compound was obtained by replacement of the C-17^3 carboxyl group with a NH_3Cl function (Fabiano et al., 1997). Aiming at mitochondrial localization within neoplastic cells, some water-soluble cationic vinyl-extended derivatives of pyro-Pheide and Chn e_6 methyl esters were prepared (Pandey et al., 1991b, 1992b). Nucleoside adducts of vinyl-substituted pyro-Pheide and Chn e_6 methyl esters were synthesized as potential anti-viral and anti-tumor drugs (Jiang et al., 1995, 1996).

Targeting. In an attempt to enhance the delivery and selectivity of the active ingredient, the Pheide was enclosed within β-cylcodextrin dimers (Roehrs et al., 1995), or was adsorbed either in monomer form on microcrystalline cellulose (Zeug et al., 2002) or as photodegradable dendrimer conjugates (Hackbarth et al., 2001).

2. Pre-clinical Studies and Efficacy

a. In Vitro Studies

Using Pheide as photosensitizer (IC_{50} 0.5–2.0 μM, 5 J cm^{-2}, 670 nm) on human pancreatic (Hajri et al., 1999) and colon (Hajri et al., 2002) carcinoma cells gave superior therapeutic results than with Photofrin®. The cyto-phototoxicity of Pheide *a* was 20-times higher than that of haematoporphyrin derivative (HpD) (Röder, 1998). The proposed phototoxic mechanism involved both type I and type II reactions (Tanielian et al., 2001). The Pheide was localized in the Golgi apparatus of OAT 75 lung carcinoma cells (Moser et al., 1992). Pheide and its methyl ester were far more phototoxic (EC_{50} 0.3–3 μM) than the esters with longer alkyl chains using a panel of human tumor cell lines. However, the butyl ester was more active than the ethyl, hexyl, octyl or benzyl analogues (Wongsinkongman et al., 2002). In a series of mono-, di-, and tri-ol derivatives of Pheide and pyro-Pheide, the mono-ol compound was the most effective for light-induced killing of mouse colon Colo26 cells (LD_{50} 0.35 μM), and possessed low dark toxicity (Bonnett et al., 1992; Bonnett et al., 1994). The photocytotoxicity observed in EMT-6 cells for Pheide amides with alkyl chain lengths of 4–6 carbon atoms, which terminated with OH or CH_3 groups, was three orders of magnitude higher than that of Photofrin®. A significant efflux of these drugs from cells, promoted by HDL and LDL lipoproteins, may account for their fast clearance from normal tissues and low phototoxicity side effects (Dagan et al., 1995).

The insertion of Zn as a central atom in 13^2-hydroxy-Pheide *a* and Chn e_6 methyl esters decreased phototoxic activity by a factor of 5–10, and increased the dark toxicity more than 40-fold (Wongsinkongman et al., 2002).

Targeting. To enhance both drug uptake by tumor cells and subsequent delivery to lysosomes, Pheide

was incorporated into liposomes coated with monoclonal antibodies. Phototoxicity (LD_{90}) toward human bladder tumor cells was about 0.8 μM (Bergstrom et al., 1994).

To target tumor cells over-expressing receptors to plasma LDL, pyro-Pheide was covalently linked by amide bonding with cholesteryl oleate as a double anchor for the LDL lipid core. The photosensitizer-LDL was successfully incorporated into human hepatoblastoma tumor cells (Zheng et al., 2002).

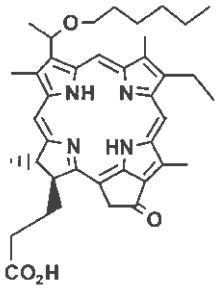
b. In Vivo studies

Twenty-four hours after i.v.-administration, the major target of Pheide *a* in rats carrying acinar pancreatic tumor was the reticuloendothelial system, with a very low level of Pheide in the skin. The ratio of tumor-tissue/surrounding-tissue partitioning of the drug was 6.7–13.5, resulting in PDT-induced (100 J cm^{-2}, 660 nm, 24 h after 9 mg kg^{-1} i.v.) selective necrosis of the tumor (66% cure, 120 days) (Aprahamian et al., 1993; Evrard et al., 1994). The higher selectivity and depth of sensitization provided by Pheide *a*, enabled more efficient PDT and better results than with 5-aminolevulinic acid (ALA)-induced protoporphyrin IX (100 J cm^{-2}, 670 nm, 24 h after 30 mg kg^{-1} i.p.) in treatment of a pancreatic tumor in mice (Hajri et al., 1999). However, the poor uptake and insufficient selectivity of Pheide in HT29 colon cancer implied that photodynamic treatment with this pigment is less safe than with Photofrin® which has better tumor uptake (Hajri et al., 2002).

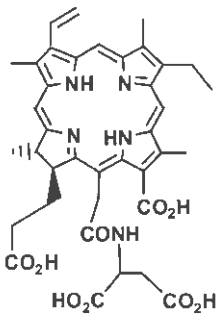
The 3-(1-alkoxyethyl) ether derivatives of Pheide *a* methyl ester and the Chn e_6 trimethyl ester showed that hydrophobic ethers were more efficient photosensiters than the parental Pheide *a*, Chn e_6 methyl esters and Photofrin®, when tested on SMT/F tumors in mice. These findings suggested differences in localization and subcellular distribution of the different drugs. The new derivatives, in particular, had a shorter lifetime in normal tissues and were excreted within 5 days after injection, compared with weeks for Photofrin®. The 3-(1-hexyloxyethyl) ether of the Pheide methyl ester showed strong photodynamic efficiency (140 J cm^{-2}, 667 nm, 24 h after 1 mg kg^{-1} i.p.), providing 50% tumor response at day 30, which is much better than obtained with the related Chn e_6 derivative, in which the isocyclic ring E is cleaved (Pandey et al., 1991a).

Pyro-Pheide *a* derivatives provided even slightly higher efficiencies. The 3-(1-hexyloxyethyl) and 3-(1-heptyloxyethyl) ethers of pyro-Pheide *a* methyl ester and related 13^1-deoxy derivatives showed 50% tumor response at day 30 under lower drug dose (0.3 mg kg^{-1}), with only minor skin photosensitivity (Pandey et al., 1992c). In the authors' opinion, the shorter-chain pyro-Pheide ethers showed diminished activity due to rapid clearance from plasma and tissues. Introduction of a double bond into the hexyl side chain (the *cis*- and *trans*-3-hexenyl ethers) essentially negated the activity (Pandey et al., 1996c). [13^1-thione]- and [3-formyl]-pyro-Pheide and Pheide methyl esters were active when illuminated at 3 h, but not 24 h, post i.p.-injection (Pandey et al., 1991a, 1992c). The 3-(1-hexyloxyethyl) and 3-(1-heptyloxyethyl) ethers of pyro-Pheide carboxylate, prepared by saponification, were as efficient as the corresponding methyl esters. The low activity of the corresponding Chn e_6 ethers probably resulted from their enhanced hydrophilicity due to enzymatic de-esterification (Pandey et al., 1996c). The half-life time of [3-(1-hexyloxyethyl)]-pyro-Pheide (code name HPPH, see Fig. 1) in rat serum was 25 h (Lobel et al., 2001) and similarly 26.9 h in dogs (Payne et al., 1996), which is longer than in mice (bi-exponential decay: 0.69 h and 21 h) (Bellnier et al., 1993). Both direct effects on the tumor cells and indirect effects via vascular damage contributed to the overall PDT response (Bellnier et al., 1993; Henderson et al., 1997). HPPH-PDT was estimated in the rat model as a useful adjuvant treatment of malignant gliomas (Lobel et al., 2001), as an effective treatment of canine oral (McCaw et al., 2000), feline facial (Magne et al., 1997) and hamster cheek pouch (Furukawa et al., 1996) squamous cell carcinomas, but as an undesirable adjuvant therapy to surgery of canine hemangiopericytomas (McCaw et al., 2001). Photodynamic activity of the anionic pyro-Pheide derivatives was correlated with intracellular localization, which, in turn, was influenced by the aggregation state of the compound upon cellular uptake (Geze et al., 1993; Macdonald et al., 1999; Matroule et al., 1999, 2001; Kelbauskas and Dietel, 2002; Sun and Leung, 2002). Amphiphilicity could play a more dominant role than lipophilicity (Macdonald et al., 1999; Kelbauskas and Dietel, 2002). However, the optimal photodynamic activity could originate from the binding of the photosensitizer to mitochondrially-located peripheral benzodiazepine receptors (Dougherty et al., 2002).

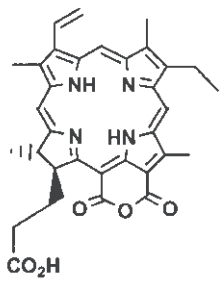
PDT with Pheide 4-hydroxybutylamide (380 J cm^{-2}, 673 nm, 1 h after 2 mg kg^{-1} i.v.) reduced the time for doubling of tumor volume to ~12 days in



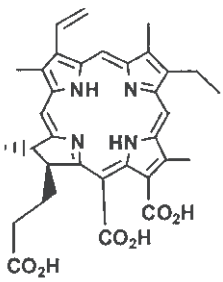
3^1-(1-hexyloxy)phytochlorin
Photochlor® (HPPH)



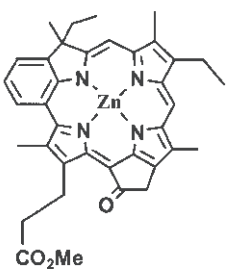
$3^1,3^2$-didehydro-rhodochlorin-15-(2-(N-2-succinyl)acetamide
NPe$_6$



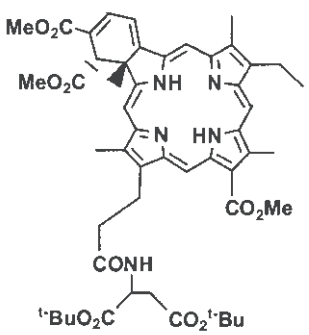
$3^1,3^2$-didehydro-15-carboxy-rhodochlorin-$13^1,15^1$-anhydride
PP *18*



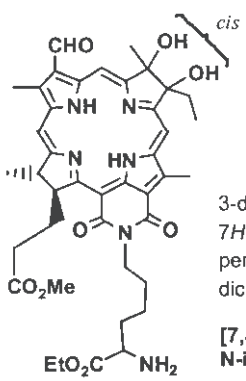
$3^1,3^2$-didehydro-rhodochlorin-15-carboxylic acid
Chn p_6



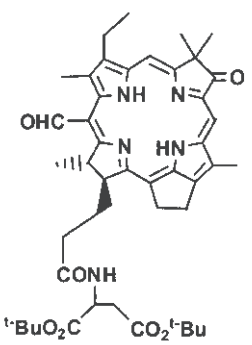
3-methyl-13^1-deoxo-13^2-oxo-benzo[*at*]-phytoporphyrinatozinc(II)
[Zn]-Benzochlorin derivative



$2^1,2^2,13$-trimethoxycarbonyl-2-methyl-2*H*,2^1*H*-benzo[*b*]-pyrrochlorin 17^3-[2-succinato-di-*tert*-butyl ester]amide
2,3-Benzoporphyrin derivative



3-desethyl-3-formyl-7,8-*cis*-dihydroxy-7*H*,8*H*-N-(5-amino-5-ethoxycarbonyl-pentyl)pyrrochlorin-$13^1,15^1$-dicarboxamide 17-methyl ester
[7,8-*vic*-dihydroxy]-BPP *18* N-imide derivative



7-methyl-8-desethyl-8-oxo-13^2-desoxo-20-formyl-phytochlorin-17^3-[2-succinato-di-*tert*-butyl ester]amide
Keto-BChn derivative

Fig. 1. Structures of some Chl derivatives used as active photosensitizers (IUPAC-approved names are followed by the trade names or abbreviations)

mouse EMT-6 tumors (Morliere et al., 1998), probably by affecting the tumor vascular integrity (Chapman et al., 1994; Dagan et al., 1995; Gatt et al., 1996). When Pheide 4-hydroxybutylamide was used, photoinactivation of erythrocytes infected by transfusion-transmissible parasites was successful in whole blood with no side effects (Grellier et al., 1997).

Water-soluble carboxymethyl- and hydroxyethyl-substituted pyro-Pheide amides of glycine or aspartic acid exhibited fast clearance from normal organs and serum but were usefully retained in tumors for 24 h in hamsters with nitrosamine-induced pancreatic cancer (Ando et al., 1991b).

Targeting. Negatively charged phospholipid vesicles, used for solubilizing [3-(1-alkoxyethyl)]-Pheide methyl ester, decreased the possibility of occlusive vascular damage (Mayhew et al., 1993). On the other hand, PDT on murine mammary tumor, performed with a short light-drug interval (15 min) using the indium complex of pyro-Pheide methyl ester (code name MV6401) in cationically-charged egg yolk phosphatidylcholine emulsion (0.018-0.072 mg kg^{-1}, 5 J cm^{-2}, 664 nm), caused blood flow stasis and vascular hyperpermeability that became apparent 3 h later (Dolmans et al., 2002b). The plasma half-life of MV6401 was ~20 min, and the drug was confined to the vascular compartment during the first 15 minutes after administration. However, neovessel formation and tumor regrowth were observed 3 days after the treatment. Two equal MV6401 doses injected 4 h and 15 min before light exposure allowed the drug to localize in both vascular and tumor cell compartments: such double administration of drug before PDT resulted in a profound delay of tumor growth and a more extensive antivascular effect (Dolmans et al., 2002a).

Combining a PDT protocol, based on administration of ALA and a Pheide derivative, synergistically enhanced the inhibition of tumor growth (lymphoma and squamous cell carcinoma) in mice (Jin et al., 2000).

D. Chlorin e_6 and Derivatives

1. General Description and Chemistry

Chn e_6, formed by anaerobic alkaline hydrolysis of Pheide *a*, is a tricarboxylic water-soluble derivative absorbing light at 654 nm and has a ϕ_Δ of about 0.7 (Spikes and Bommer, 1993; Zenkevich et al., 1996; Fernandez et al., 1997). $[Sn]^{IV}$-Chn e_6 absorbs at 632 nm and generates singlet oxygen with ϕ_Δ equal to 0.83 (Spikes and Bommer, 1993). Amides of Chn e_6 at C-13[1] were regioselectively obtained by aminolysis of the isocyclic ring (Ando et al., 1991a, 1992; Gurinovich et al., 1992; Zhang and Xu, 1999; see also Ma and Dolphin, 1996; Belykh et al., 2002). Mono-L-aspartyl Chn e_6 (code name NPe_6, Fig. 1), obtained by amidation of the acetic acid residue of Chn e_6 with L-aspartic acid (Gomi et al., 1998), possessed an increased hydrophilicity (Boyle and Dolphin, 1996) and a ϕ_Δ of 0.77 (Spikes and Bommer, 1993).

2. Pre-clinical Studies and Efficacy

a. In Vitro Studies

A possible role for the acidic tumor microenvironment in the preferential uptake and retention of Chn e_6 has been reported (Zorin et al., 1996; Cunderlikova et al., 1999, 2000; Shevchuk et al., 2002). Higher accumulation of Chn e_6 dimethyl ester in leukemic cells caused their selective photocytolysis (Savitskiy et al., 2002). A glucosamine salt of Chn e_6 revealed high antifungal activity (Strakhovskaya et al., 2002).

NPe_6 (Fig. 1), localized in the lysosomes of murine hepatoma cells, caused photodamage by triggering the mitochondrial apoptotic pathway by releasing lysosomal proteases (Reiners et al., 2002). Serum components inhibited cellular uptake of NPe_6, and only free pigment was accumulated by murine leukemia cells and caused phototoxicity (Sheyhedin et al., 1998).

Targeting. Triacetoxymethyl ester of Chn e_6 (CAME) and acetoxymethyl ester of Pheide *a* (PAME) were synthesized as photosensitizers for lysosome-mediated PDT (Sahai et al., 1993). These lipophilic esters, reaching acidic lysosomes by endocytosis, were hydrolyzed by esterases into pH-sensitive amphipathic compounds. Contact with the neutral pH of the adjacent cytosol results in conversion of the hydrophobic drug to a more hydrophilic anionic species, presumably by allowing its diffusion into the lysosomal compartment and partitioning throughout the lipophilic and aqueous compartments of the cell. Brief incubation of murine leukemia cells with 10 mM CAME followed by irradiation, led to mitochondrial photodamage and apoptosis, whereas higher doses of CAME inhibited apoptosis, with cell death probably occurring via necrosis (Kessel and Poretz, 2000).

Immunoconjugates of Chn e_6 with anti-ovarian carcinoma murine monoclonal antibody were more

effective in the selective photochemical eradication of target cells than the free pigment (Goff et al., 1991, 1992).

Light-dependent killing of mammary adenocarcinoma cells was examined with a Chn e_6-transferrin conjugate, which was aimed at a corresponding receptor that is highly expressed in tumor cells (Cavanaugh, 2002).

Targeted delivery of $[Sn]^{IV}$-Chn e_6, conjugated to epidermal growth factor (EGF) through a carrier, showed increased phototoxicity to squamous carcinoma cells in relation to phototoxicity of the non-conjugated sensitizer, which express an increased number of EGF-receptors; however, the affinity of the conjugate was strongly dependent on the carrier used (dextran, polyvinyl alcohol or human serum albumin) (Gijsens and de Witte, 1998; Gijsens et al., 2000). $[Sn]^{IV}$-Chn e_6 linked to IgG selectively killed otherwise resistant strains of *Staphylococcus aureus* (Embleton et al., 2002).

The importance of positive charges on the sensitizer's periphery to the overall photodynamic activity was demonstrated by the killing of both gram-positive and gram-negative bacteria by cationic conjugates of Chn e_6 (Soukos et al., 1998; Hamblin et al., 2002b).

Conjugates for doubly-targeted delivery to both cell and nucleus were constructed using Chn e_6 together with BSA as a carrier, insulin as internalizable ligand and viral T-antigen within a β-galactosidase fusion protein as a nuclear localization factor. This molecular assembly demonstrated a 2400-fold higher photodynamic activity in human hepatoma cells (EC_{50} of 0.13 nM) compared to free Chn e_6 (Akhlynina et al., 1997). Co-incubation with adenovirus additionally increased the nuclear photosensitizing activity (Akhlynina et al., 1999). Chn e_6 was also attached to linear or branched peptides for guidance to both cytoplasmic and nuclear targets: such conjugates displayed 400- and 40-fold more phototoxicity in CHO and RIF-1 cells than Chn e_6 alone (Bisland et al., 1999).

b. In Vivo Studies

Photodynamic treatment of rat M-1 sarcoma with Chn e_6 (90 J cm^{-2}, 647 and 676 nm, 3 h after 5–10 mg kg^{-1} i.p.) induced tumor necrosis to a depth of 14–16 mm via simultaneously damaging both vascular stroma and malignant cells. The effect was potentiated by increasing the drug and light doses, but was attenuated with the extension of the drug-to-light interval (Kostenich et al., 1991): photoradiation of various transplantable rat tumors was far more effective 15 min after injection than after 24 h, leading to 100% cure on day 14 after the treatment of spindle cell fibrosarcoma and alveolar liver cancer (Kostenich et al., 1993). The regression was correlated with the morphological difference between the tumor microcirculatory bed, as the main target of photodynamic exposure, and the normal one (Kostenich et al., 1993). Pharmacokinetic studies revealed a maximum concentration of Chn e_6 in blood, internal organs and muscle 3 h after i.p.-injection, and in tumors after 12–18 h. Increased content and the relatively long clearance time of Chn e_6 (>72 h) in blood and tumor were attributed to its binding to and interaction with blood components including erythrocytes and transport proteins (Kostenich et al., 1994). Nevertheless, Chn e_6 demonstrated a high antitumor efficacy in colon carcinoma in mice with minimal damage to normal tissues (Orenstein et al., 1996). The efficient application of Chn e_6-PDT for treating rheumatoid arthritis has also been discussed (Tauraytis et al., 1992).

The water-soluble amide formed from Chn e_6 and ethylenediamine stimulated affinity for tumor cells and cellular membranes. The photodynamic effect, when the maximal concentration of sensitizer was reached in the tumor, was higher with the Chn e_6-amide than with Chn e_6, possibly due to preferential binding with lipoproteins (Gurinovich et al., 1992).

NPe_6 rapidly clears from the body (Ferrario et al., 1992). The maximal PDT effectiveness in murine mammary tumors, achieved following light treatment 2 h after drug injection, was correlated with the plasma, not tumor, levels of the photosensitizer (Ferrario et al., 1992) which possesses a selective affinity for proliferating neovasculature (Roberts and Hasan, 1992). In uterine cervical carcinoma the PDT effect using NPe_6 resulted from tumor necrosis secondary to the obstruction of blood vessels around the tumor (Nakamura et al., 2002). In rats bearing chondrosarcoma, the achieved tumor cure of 83%, with no regrowth for 21 days, was found after an optimal delay of 4 h between injection and illumination (i.e. 135 J cm^{-2}, 664 nm, 4 h after 10 mg kg^{-1} i.v.), when the effect of both vascular stasis and direct tumor cytotoxicity was maximal (McMahon et al., 1994).

Atherosclerotic plaques of abdominal aorta can be selectively degraded by NPe_6-PDT, as demonstrated in cholesterol-fed rabbits (50–200 J cm^{-2}, 664 nm,

6 h after 5 mg kg^{-1} i.v.) (Saito et al., 1998). Two main mechanisms might be complementary and synergistic in the photodynamic production of vascular lesions: endothelial cell damage and platelet aggregation (fibrin plugging) (Yamamoto et al., 1999).

Choroidal vessel occlusion was evident starting from 2.65 J cm^{-2} in pigmented rabbits and 0.88 J cm^{-2} in non-pigmented rabbits (2 mg kg^{-1} of NPe_6 i.v.). Lesion diameter decreased as the time between injection and irradiation increased from 5 min to 24 h (Kazi et al., 2000). NPe_6-PDT was found to have a lower threshold for choroidal vessel occlusion than for retinal vessels (Mori et al., 1999; Peyman et al., 2000a); thus, the PDT effect varied depending on the target characteristics (Peyman et al., 2000b).

Targeting. Both immunoconjugates of Chn e_6 with anti-ovarian carcinoma murine monoclonal antibody and non-conjugated photosensitizer reached peak tumor concentrations at 24 h but the absolute concentrations of the conjugate were always 2 to 3-fold higher. Also, after 24 h, the conjugate concentrations were 3.5- to 7.2-fold higher in tumor than in non-tumor cells. PDT with a single light exposure demonstrated a dose-dependent relationship with the fluence and the conjugate concentration. However, there was significant treatment-related toxicity at all light fluences tested (Goff et al., 1994).

A conjugate of Chn e_6 with BSA was efficiently taken up by a scavenger pathway, localized in areas of the intimal hyperplasia of the rat's abdominal aorta, and functioned as a PDT-sensitizer (Nagae et al., 1998). Covalently linked Chn e_6-albumin conjugate showed superior PDT-activated tissue sealing of scleral incisions than did non-covalent mixtures (Khadem et al., 1999).

Bio-distribution studies showed that the polyanionic conjugates of Chn e_6 with murine monoclonal antibody accumulated more selectively in liver tumors than corresponding polycationic conjugates or free Chn e_6. PDT with the polyanionic conjugates was more effective in eradicating liver tumors and resulted in better survival of normal tissue (Del Governatore et al., 2000b). Chn e_6-succinylated polylysine conjugate was used to activate the transforming growth factor P that modulates cartilage metabolism in osteoarthritis (Sullivan et al., 2002). A cationic poly-L-lysine conjugate of Chn e_6 to a Fab´ fragment of the murine monoclonal anti-human ovarian carcinoma antibody, showed a PDT response better than that of the anionic conjugate or free Chn e_6, with no systemic toxicity from the treatment (Molpus et al., 2000). The cationic conjugate, in combination with *cis*-platin chemotherapy ex vivo, displayed a synergistic effect (Duska et al., 1999). A comparison between the positively-charged polylysine- and negatively-charged succinylated polylysine-conjugates of Chn e_6 for PDT of rat orthotopic prostate tumor models indicated that both conjugates initially bind to the endothelium lining of the vasculature. However, the anionic compound extravasated faster into the tissue (Hamblin et al., 1999). Targeted cationic conjugates showed superior binding to human colon cancer cells (Del Governatore et al., 2000a). The use of cationic poly-L-lysine conjugate of Chn e_6 to control wound healing was shown in mice (Hamblin et al., 2002a).

The use of water-soluble N-(2-hydroxypropyl) methacrylamide (HPMA) polymer for anticancer drug delivery was intended to bypass some forms of multidrug resistance, to enhance preferential accumulation in tumor tissue by increased permeability and retention, and to take advantage of new targeting strategies such as polymerizable antibody fragments and synthetic receptor-binding epitopes (Kopecek et al., 2001). Combination therapy with the HPMA-anticancer drug containing doxorubicin and meso-Chn e_6 attached via an enzymatically degradable oligopeptide, cured tumors which could not be cured with either chemotherapy or PDT alone. HPMA-copolymer-bound drugs exhibited selective tumor accumulation in contrast to free drugs. Most effective was the combination of multiple chemotherapy with HPMA-copolymer-doxorubicin and multiple PDT with HPMA-copolymer-meso-Chn e_6, especially with the attachment of monoclonal antibodies to both copolymers. Reduced non-specific toxicity has been a constant advantage in all the preclinical studies (Kopecek et al., 2001).

E. Purpurin 18, Chlorin p_6, and Derivatives

1. General Description and Chemistry

Purpurin *18* (PP *18*, Fig. 1), having a 6-membered anhydride ring as a result of oxidative cleavage of isopentanone ring E, is easily prepared from Pheide *a* and its esters (Kenner et al., 1973; Hoober et al., 1988) or directly from a crude extract of Chl *a* (Mironov et al., 1993). PP *18* is lipophilic, has a strong absorption at 700 nm and ϕ_Δ of about 0.7 (Zenkevich et al., 1996). PP *18,* a stable intermediate, can be used for further chemical transformations. Alkaline cleavage

of the anhydride ring produces water-soluble Chn p_6 (Fig. 1), which absorbs at 656 nm and provides ϕ_Δ of about 0.6 in ethanol, the same as Chn e_6 (Zenkevich et al., 1996). The opening of the anhydride ring of the PP *18* methyl ester by L-lysine as a nucleophile yielded a water-soluble Chn p_6 13^1-lysylamide methyl ester (K. M. Smith et al., 1992; Lee et al., 1993). Replacement of the 3-vinyl group with the 1-hydroxyethyl and 1,2-dihydroxyethyl functions did not cause significant spectral changes, but substantially increased the hydrophilicity of the corresponding Chn p_6 derivatives. In contrast, the distinct bathochromic shift with vinyl oxidation to acetyl (25 nm) and formyl (35 nm) groups did not influence the amphiphilicity balance (Kozyrev et al., 1994; Pandey et al., 1994). The propionic acid residue of PP *18* was converted into corresponding amides with alanine and aspartic acid (Mironov et al., 1993).

Partial reduction transformed the anhydride ring of PP *18* into a δ-lactone. The mixture of the two products and their 3-(1-hydroxyethyl) and 3-acetyl derivatives displayed a higher stability toward acidic and basic cleavage; however, their spectral bands in the red region were blue shifted by 22–37 nm (Mironov et al., 1998).

PP *18* cyclic imides, isoimides, and the corresponding Chn p_6 derivatives with 1-hexylamine (Pandey et al., 1994; Kozyrev et al., 1996c) and N,N-dimethylethylenediamine (Kozyrev et al., 1997) instead of lysine, have also been synthesized. Following a similar QSAR-investigation approach as with Pheides, a set of lipophilic PP *18* N-imide derivatives was synthesized with various alkyl, aryl, and fluoroaryl substitutions at the C-3, C-8, C-20 and N-13 positions of the macrocycle: the main Q_y absorption band of this set of compounds was near 700 nm and they had ϕ_Δ values of 0.57–0.60 (Rungta et al., 2000; Zheng et al., 2000b, 2001b; Gryshuk et al., 2002).

PPs of increased hydrophiliciy, comprising cyclic imides with NH, N-substituted residues of C_2-C_4 aliphatic alcohols, carboxylic acids (C_1, C_5) or containing the N-OR group, where R is a hydrogen, alkyl or acyl residue, have their major absorption in the 706–718 nm domain. The replacement of the vinyl group by a formyl function resulted in a red shift to 750 nm (Mironov and Lebedeva, 1998; Mironov et al., 1999). A PP derivative with N-(3-hydroxypropyl) residue absorbs at 711 nm and has a ϕ_Δ value of 0.66 (Feofanov et al., 2002).

A positively charged PP was recently prepared by introducing a pyridinium group at the C-5 position of [Ni]-Chn p_6 trimethyl ester or at the C-12^1 position of [Ni]-PP *18* N-hexylimide methyl ester (Mettath et al., 2000).

2. Pre-clinical Studies and Efficacy

a. In Vitro Studies

The photodynamic efficacy of [3-formyl]-Chn p_6 and Chn p_6 were similar in causing necrotic death of A-549 human adenocarcinoma cells (IC_{50} about 4-8 μM), with the mitochondria as a primary target. The water-soluble Chn p_6 13^1-lysylamide methyl ester was found photocytotoxic against 9L glioma cells at concentrations 10 to 100-fold lower than Photofrin® (K. M. Smith et al., 1992; Lee et al., 1993). The Chn p_6 lysylamide was localized by endocytosis in the endosomal compartment, causing morphological damage in the mitochondria, Golgi apparatus, and rough endoplasmic reticulum (Leach et al., 1993).

A hydrophilic PP N-(3-hydroxypropyl)imide demonstrated photocytotoxicity on A549 human adenocarcinoma cells 60-fold higher than [3-formyl]-Chn p_6, with no dark cytotoxicity. Subcellular localization implied mitochondria and Golgi apparatus as the primary targets (Feofanov et al., 2002).

b. In Vivo Studies

PP *18* was found inactive, probably due to the instability of the anhydride ring in vivo (Zheng et al., 2000a). PDT on Ehrlich carcinoma in mice (90 J cm^{-2}, 696 nm, 5-10 mg kg^{-1} i.v.), performed 1-6 h after [3-formyl]-Chn p_6 administration, resulted in severe complications and light-induced deaths, whereas after 24 h it induced moderate suppression of tumor growth (Grichine et al., 2001).

PDT with Chn p_6 lysylamide on 9L glioma tumors in rats (100 J cm^{-2}, 664 nm, 4 h after 2.5 mg kg^{-1} i.v.) showed long-term inhibition of tumor growth. However, side effects in normal tissues, including severe skin necrosis, were observed (Leach et al., 1992). To prevent conversion of Chn p_6 lysylamide into PP imide, the C-15^1 acid residue was esterified (Lee et al., 1993). The Chn p_6 lysylamide esters were less efficient PDT sensitizers than the corresponding PP imide when tested on mammary adenocarcinoma in mice. However, these lysylamide agents caused a substantial direct toxicity with 5 mg kg^{-1} dose in contrast to NPe_6 (Kessel et al., 1995).

The tumorcidal ability of the 3-(1-alkoxyethyl)

ethers of PP *18* N-hexylimide methyl ester compared with that of the 17^3-alkylamide derivatives of PP *18* N-hexylimide was studied by Zheng et al. (2000b); the therapeutic response of RIF tumors in mice (135 J cm^{-2}, 705 nm, 24 h after 1 μmol kg^{-1}) was stronger with the ether derivatives, especially with the *n*-hexyl chain (100% tumor response on day 30), than with the amides. The location of alkylether substituents in alkylether analogues of meso-PP *18* N-hexylimide methyl ester and meso-PP *18* N-decylimide methyl ester is important for photosensitizing efficiency: the 3-(1-heptyloxyethyl is more efficient than 8-(1-heptyloxyethyl), 3-octyloxymethyl and 20-heptyloxymethyl derivatives (Rungta et al., 2000). Essential differences in tumor uptake, selectivity and PDT efficacy were observed among homologues of the PP-*18* imide methyl ester containing 3-(1-O-alkyl) and 13^2-N-alkyl substituents possessing similar lipophilicity and singlet oxygen production, suggesting differences in their pharmacokinetics and pharmacodynamics (Zheng et al., 2001b). Screening of PP *18* imide methyl esters, containing aromatic (3,5-dimethylbenzylic) or trifluoromethyl-aromatic substituents instead of O-alkyl or N-alkyl groups, indicated the superiority of the fluorinated purpurinimides, especially the N-fluoro-aryl derivatives (Gryshuk et al., 2002).

PDT in P-388 leukemia-bearing mice with hydrophilic PP N-(3-hydroxypropyl)imide (230 J cm^{-2}, >690 nm, 24 h after 25 mg kg^{-1}) halved the tumor growth rate (Mironov et al., 1999).

Targeting. Meso-PP *18* imide methyl ester, N-substituted with galactose or lactose via diene spacer, was designed for targeted delivery to the galectin-1 receptor, which is highly expressed by malignant cells. Preliminary studies have indicated the superiority of carbohydrate conjugates over non-conjugated photosensitizers (Zheng et al., 2001a).

F. Benzochlorin Derivatives

1. General Description and Chemistry

Benzoisobacteriochlorin, formed by the cyclization of a meso-acrolein to a β-pyrrolic position in the [20-(2-formylvinyl)-13^1-deoxypyro]-[Ni]-Pheide methyl ester (Vicente and Smith, 1991), was converted into benzochlorin, and then to 5-formyl, alkoxymethyl and cationic iminium salt derivatives (Pandey et al., 1995) and also to 13^1, 13^2-oxobenzochlorins (Fig. 1) (Mettath et al., 1998). These compounds have absorption maxima between 708-759 nm. Similarly, based on the PP *18* N-hexylimide methyl ester, 3-ethylidene-substituted isobacteriochlorins and fluorinated benzochlorin (λ_{max} 751 nm) were produced (Mettath et al., 1999).

G. Diels-Alder Adducts of Chlorins

1. General Description and Chemistry

When the 3-vinyl group of Pheide *a* was utilized as a diene component, isomerically pure 2,3-benzoporphyrin analogues (Fig. 1) were obtained (Pandey et al., 1993, 1996b; Ma and Dolphin, 1997). Also, by Diels-Alder cycloaddition, an 8-vinyl-meso-PP *18* methyl ester was transformed into 7,8-benzobacteriopurpurins, having an absorption at 760-795 nm (Zheng et al., 1996). Regioselective cycloaddition of diazomethane to pyro-Pheide and PP *18* N-methyl imide produced the corresponding 1′-pyrazolinyl-substituted derivatives, which were altered to cyclopropyl derivatives by pyrolysis (Kozyrev et al., 2003). However, with [13^2-oxo]-pyro-Pheide *a*, the primary target of diazomethane was the α-diketone ring, producing verdinochlorins with fused cyclohexenone rings and an absorption at 739–777 nm (Kozyrev et al., 1998a).

2. Pre-Clinical Studies and Efficacy

a. In Vivo Studies

In preliminary tests, a benzporphyrin derivative of rhodoporphyrin XV aspartyl amide (λ_{max} 668 nm) was a successful photosensitizer against SMT/F tumors in mice at 3 h but not 24 h after injection of 1 mg kg^{-1} (Pandey et al., 1996b).

H. Glycol- and Ketobacteriochlorin Derivatives

1. General Description and Chemistry

The reaction of non-vinyl derivatives of pyro-Pheide *a*, Chn e_6, PP *18* and its N-imides with osmium tetroxide yielded *vicinal 7,8*-dihydroxy-substituted bacteriochlorins (BChns, Fig. 1) with reduction of opposite pyrrole rings B and D: these compounds show significant spectral red shifts up to 754 nm for the 3-formyl-derivatives of pyro-Pheide *a* and Chn e_6 and 828 nm for the 3-formyl-derivative of

[Zn]-PP *18* (Pandey et al., 1992a,c, 1994, 1997b; K. M. Smith et al., 1992; Kozyrev et al., 1994, 1996a). By a similar approach, [Ni]-complexes of meso-Chn e_6 and meso-PP *18* methyl esters yielded *vicinal*-dihydroxy-substituted isoBChlns with reduction of adjacent pyrrole rings (Kozyrev et al., 1996a).

The pinacole-pinacolone rearrangement in *vicinal*-dihydroxy-BChns led to keto-BChns (Fig. 1). The migration of alkyl groups and the fluctuation of absorption maxima (711-786 nm) of the products obtained was influenced by the number and position of the electron-withdrawing groups (Pandey et al., 1992a, 1997b; Kozyrev et al., 1996b). The keto-BChn from [13^1-deoxo]-mesopyro-Pheide *a* provided a ϕ_Δ of 0.40 (Pandey et al., 1997a).

2. Pre-Clinical Studies and Efficacy

a. In Vivo Studies

BChn products derived from pyro-Pheide *a* and Chn e_6 were photodynamically inactive (135 J cm^{-2}, 702 nm, 3 h after 0.3 mg kg^{-1} i.p. or 4 and 24 h after 10 mg kg^{-1} i.v.) (Pandey et al., 1992c; Kessel et al., 1993). Among [3-formyl]-bacteriopurpurin (BPP) agents, however, the N-13^2-lysylimide and C-17^3-aspartylamide derivatives, irradiated 3 h post i.v.-injection (135 J cm^{-2}, 815 nm, 5 mg kg^{-1}), exhibited 100% tumor response on day 7, while the lysyl-free [3-formyl]-BPP was inactive (Pandey et al., 1994).

An isomeric mixture of keto-BChns, obtained from [3-acetyl-8-isobutyl]-pyro-Pheide *a* methyl ester, was successful in treating mice bearing the RIF tumor (135 J cm^{-2}, 5–10 mg kg^{-1}) when photoexcited at 4 h but not 24 h after i.v.-administration; however, some post-PDT toxicity was observed. The efficacy and cytotoxicity were correlated with plasma rather than with the tumor levels of the drug (Kessel et al., 1993). Similarly, keto-BChns from [13^1-deoxo-20-formyl]-mesopyro-Pheide *a* were photodynamically active on SMT/F tumors in mice (135 J cm^{-2}, 1–2.5 mg kg^{-1}), when illuminated 3 h but not 24 h after i.v.-administration, but post-PDT mortality was recorded. Di-tert-butylaspartic esters were more potent than the methyl ester derivative, probably due to esterase activity. Cutaneous phototoxic side effects were not observed from day 9 after keto-BChn injection. Photosensitizing efficacy was correlated with an affinity for the peripheral benzodiazepine receptor (Pandey et al., 1997a).

I. Dimeric and Oligomeric Structures

1. General Description and Chemistry

A number of Chl-based dimers and trimers with ether, amide and carbon-carbon linkages were prepared (Ando et al., 1991a, 1992; Brandis et al., 1992; Jaquinod et al., 1996; Pandey et al., 1996d; Zheng et al., 2000a) similar to Photofrin®, which consists of a mixture of dimers and higher oligomers with ether, ester and carbon-carbon linkages determining its biological activity (Pandey and Zheng, 2000; Macdonald and Dougherty, 2001). Dimers of pyro-Pheide *a*, Chn e_6 and PP *18* derivatives, linked with amide bonds via lysine and propylenediamine, had a $\phi_\Delta < 0.5$.

2. Pre-Clinical Studies and Efficacy

a. In Vivo Studies

Dimers of pyro-Pheide *a*, Chn e_6 and PP *18* derivatives, linked with amide bonds via lysine and propylenediamine, were evaluated in mice SMT/F tumors (135 J cm^{-2}, 665 nm, 24 h after 4 μmol kg^{-1} i.v.) and they generated weak antitumor activity relative to the related monomers (Pandey et al., 1996d; Zheng et al., 2000a), which contrasts with the enhanced effect of porphyrin dimers and trimers.

J. Chlorophyll Metabolites

1. General Description and Chemistry

Numerous metabolic derivatives of Chl, discovered in the last decade, exhibit biological activity as protective antioxidants or cellular signaling mediators (Ma and Dolphin, 1999). Some of them have potential in PDT. A hydrophobic Chl metabolite from silkworm excreta, 13^2-hydroxy-Phe *a*, generates singlet oxygen at a ϕ_Δ of 0.50 (Dai et al., 1992). Tolyporphin (see formula in Chapter 1, Scheer), extracted from the cyanobacterium *Tolypothrix nodosa*, has a structure similar to diketo-BChn photosensitizers: it possesses absorbance at 675 nm and has two sugar moieties, providing solubility in water (Prinsep et al., 1992).

2. Pre-clinical Studies and Efficacy

a. In Vitro Studies

Pheide *a* methyl ester isolated from *Garuga pinnata*

Roxb. leaves showed promising light-induced cytotoxicity against a number of human cancer cell lines including drug-resistant sublines (Wongsinkongman et al., 2002).

Tolyporphin was 6-, 70- and 5,000-fold more effective in photokilling EMT-6 tumor cells than Pheide *a* [4-hydroxybutylamide], [3-(1-hexyloxyethyl)]-pyro-Pheide *a* methyl ester and Photofrin®, respectively: this enhancement is probably due to permeation to different subcellular sites such as the endoplasmic reticulum and nuclear membrane and to reversal of multiple drug resistance (Morliere et al., 1998). Binding of tolyporphin to P-glycoprotein inhibited the transport of cytotoxic natural product drugs, enhancing the cytotoxicity of adriamycin or vinblastine in SK-VLB cells (C. D. Smith et al., 1994).

b. In Vivo Studies

PDT with tolyporphin (38 J cm^{-2}, 681 nm, 1 h after 2 mg kg^{-1} i.v.) appears promising as it delayed regrowth of mouse EMT-6 tumors by 20 days indicating that this sensitizer is 10-20 times more potent than Pheide *a* [4-hydroxybutylamide] or [3-(1-hexyloxyethyl)]-pyro-Pheide *a* methyl ester (Morliere et al., 1998). A very low level of the pigment in blood in contrast to elevated delivery to tumor 1 h after administration implied mutual direct (tumor cells) and indirect (vasculature) photokilling effect (Morliere et al., 1998).

III. Clinical Trials

PDT with HPPH (trade name Photochlor®) has entered Phase I/II clinical trials (Roswell Park Cancer Institute, Buffalo). Excellent results were reported in the treatment of five oesophageal cancer patients with no skin phototoxicity (Pandey, 2000). A recent study of the drug pharmacokinetics in humans demonstrated a bicompartmental clearance with short and long half lifetimes of 7.77 h and 596 h, respectively (Bellnier, 2003).

NPe_6-PDT entered a Phase I study (University of Louisville) on 11 patients with superficial cutaneous malignancies. Sixty six percent of the sites were tumor-free after 12 weeks (100 J cm^{-2}, 664 nm, 4 h after 2.5–3.5 mg kg^{-1} i.v.). At these drug doses, there was no apparent selectivity for destruction of tumor cells compared with normal skin cells, but only temporary generalized skin photosensitivity was noted (Taber et al., 1998) despite detection of the drug in the plasma for up to 6 weeks (Kessel, 1997). Phase II trials have commenced in Japan for use in endobronchial carcinoma; so far, thirty-nine lesions in 35 patients have been treated with an 85% response (33 lesions), out of which 29 patients (83%) showed a complete recovery (Furukawa et al., 2001).

Clinical trials with HPMA-copolymer-meso-Chn e_6 are also in progress (see Kopecek et al., 2001).

A clinical evaluation of PDT in different tumors with a water-soluble pigment mixture based on Chn e_6 and Chn p_6 (trade name Radachlorin®), is now being performed in Russia (RadaPharma, 2002).

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